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EFFECT OF CHEMICALS ON THE CELL MEMBRANE TRANSPORT OF
NUCLEOSIDES(U) TENNESSEE UNIV MEMORIAL RESEARCH CENTER
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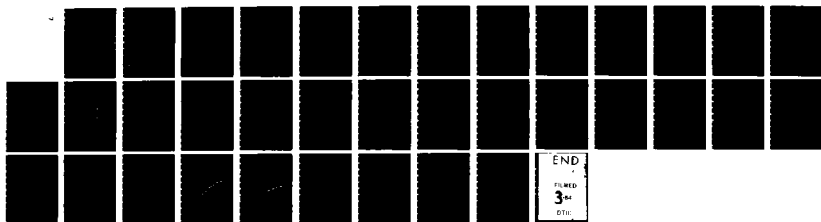
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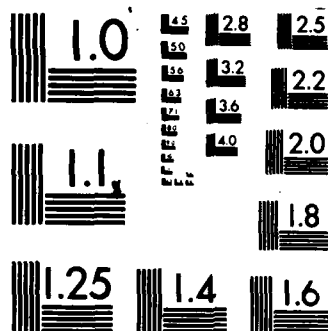
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EFFECT OF CHEMICALS ON THE CELL MEMBRANE
TRANSPORT OF NUCLEOSIDES

DEPARTMENT OF MEDICAL BIOLOGY
UNIVERSITY OF TENNESSEE MEMORIAL RESEARCH CENTER
1924 Alcoa Highway
KNOXVILLE, TENNESSEE 37920

Paul W. Wigler, Ph.D.
Professor

Controlling Office: USAF Office of Scientific Research/NL
Bolling Air Force Base, DC 20332

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) Effects of Chemicals on the Cell Membrane Transport of Nucleosides, by Paul W. Wigler, Ph.D. The apparatus and methodology for a high speed kinetic assay of purine efflux has been developed. The procedure is based on a flow system with a membrane filter to remove preloaded L5178Y cells and a sensitive rapid detector of the fluorescence emission of a buffer that contains a transport substrate, 2-aminopurine (AP). Two other purines interact rapidly with the AP carrier, hypoxanthine and uric acid. The rate of AP efflux from preloaded cells is increased by hypoxanthine in the external buffer and the efflux rate is decreased by uric acid in the buffer. Perfluorodecanoic acid (PFDA), adenine, or xanthine in the external buffer have no direct effect on the rate of AP efflux, in comparison with the controls. L5178Y cells were given a prior incubation with 200 µg/ml PFDA at 30°C for 24 hr. These cells were preloaded with 100 µM AP and the excess substrate was removed by rinsing the cells with cold buffer. The prior incubation of the cells with PFDA produces a total inhibition of the efflux of AP. On the other hand, (cont.)					
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19. (cont.) a prior incubation of the cells with PFDA plus 50 mU/ml bovine insulin produces approximately 40% inhibition in comparison with controls. These findings suggest that the purine carrier exists in an active and an inactive form; PFDA treatment inhibits formation of active carrier. Insulin appears to stabilize the active carrier and protect against the effects of PFDA. h

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103. Abstract: Effects of Chemicals on the Cell Membrane Transport of Nucleosides, by Paul W. Wigler, Ph.D.

The apparatus and methodology for a high speed kinetic assay of purine efflux has been developed. The procedure is based on a flow system with a membrane filter to remove preloaded L5178Y cells and a sensitive rapid detector of the fluorescence emission of a buffer that contains a transport substrate, 2-aminopurine (AP). Two other purines interact rapidly with the AP carrier, hypoxanthine and uric acid. The rate of AP efflux from preloaded cells is increased by hypoxanthine in the external buffer and the efflux rate is decreased by uric acid in the buffer. Perfluorodecanoic acid (PFDA), adenine, or xanthine in the external buffer have no direct effect on the rate of AP efflux, in comparison with the controls. L5178Y cells were given a prior incubation with 200 $\mu\text{g/ml}$ PFDA at 30⁰ for 24 hr. These cells were preloaded with 100 μM AP and the excess substrate was removed by rinsing the cells with cold buffer. The prior incubation of the cells with PFDA produces a total inhibition of the efflux of AP. On the other hand, a prior incubation of the cells with PFDA plus 50 mU/ml bovine insulin produces approximately 40% inhibition in comparison with controls. These findings suggest that the

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purine carrier exists in an active and an inactive form; PFDA treatment inhibits formation of active carrier. Insulin appears to stabilize the active carrier and protect against the effects of PFDA.

104. Background

The surface active perfluorocarboxylic acids are used in many commercial processes that require effective wetting agents. Aqueous foams of derivatized perfluorinated fatty acids, of different carbon chain lengths, are also useful for extinguishing accidental fires of petroleum liquids (Olson et al., 1983). Apparently, the fire-inhibitory perfluorinated mixture produces a film that may separate the petroleum fuel from atmospheric oxygen.

The relative animal toxicity of purified perfluorinated fatty acids has been investigated by the US Navy (see Olson et al., 1983). In these studies, it was observed that perfluorooctanoic acid (PFOA) produces a transient weight loss, but no mortality in young rats. By contrast, the treatment of rats with perfluorodecanoic acid (PFDA) produces a severe decrease in body weight and the PFDA-treated animals die within three weeks. These findings indicate a molecular specificity in the toxicity of these substances; the ten carbon compound may be more hazardous than the eight carbon compound.

Additional toxicology experiments, conducted by the US Air Force, have shown a 30-day LD₅₀ for PFOA treated rats approximately 5-fold higher than the corresponding value for PFDA. Furthermore, PFDA produces a delayed mortality to rats with symptoms of severe weight loss and inability to eat.

Liver tissue has been isolated from PFDA-treated rats for an analysis of the fatty acid composition (Olson et al., 1983). These experiments showed a relative increase in the unsaturated fatty acids and a relative decrease in the saturated fatty acids after PFDA-treatment (especially in the stearic to oleic acid ratio). Andersen et al., (1982) have suggested that an increase of the unsaturated lipids in the composition of the cell membrane may produce an increase in the membrane fluidity. The effect of PFDA on membrane fluidity of several cells and tissues is currently under investigation by M. George and M.E. Andersen at Wright-Patterson AFB, Ohio. These authors have also observed that erythrocytes treated with PFDA in vivo are relatively resistant to osmotic effects; these findings are consistent with an increase in membrane fluidity for PFDA-treated red cells.

Although liver cells seem most susceptible to PFDA, several other tissue

cells also show morphological changes after PFDA treatment (Olson et al., 1983). The structure of the lymphocytes isolated from PFDA treated rats is different from untreated controls. To further investigate the effects of PFDA on lymphocytes, Andersen et al., (1982) studied a mouse lymphocyte cell line (L5178Y) in cell culture. Preliminary studies by the foregoing authors indicate that sublytic concentrations of PFDA seem to have an insignificant effect on the growth of L5178Y cells in suspension culture. On the other hand, PFDA treatment may influence clone-forming capacity when the L5178Y cells are plated in semi-soft agar. This phenomenon is currently under further investigation by M. George and M.E. Andersen.

Experimental results of the PFDA toxicity to living animal cells suggest that the primary site of toxic action is on the metabolism of the lipids in the cells. These metabolic changes may produce a modification in the ratio of unsaturated to saturated phospholipids in the cell membrane. The phospholipids of the cell membrane provide a hydrophobic barrier to the simple diffusion of polar substances through the cell membrane. Furthermore, the membrane phospholipids provide a supporting matrix for the protein transporters of polar substances. Pritchard (1979) has suggested that DDT, a lipid-soluble organochlorine compound, reacts with the lipid surrounding the sodium ion transporter in the cell membrane. The proposed DDT-lipid interaction may explain the noncompetitive inhibition of the sodium transporter (sodium pump) by DDT, that was observed by Pritchard.

The DDT inhibition of the sodium pump suggests that membrane lipid changes caused by PFDA treatment could produce a delayed inhibition of a carrier system in the membrane of L5178Y cells. This is the rationale that led to an investigation of the PFDA inhibition of the efflux of 2-aminopurine with L5178Y cells.

A sample of L5178Y cells was shipped by M. George to this laboratory on May 9, 1983; this subline of L5178Y originated from the laboratory of C.F. Arlett (see Cole and Arlett, 1976). The cells were cultured for two weeks to provide enough cells for transport studies. Preliminary experiments on the effects of PFDA-treatment on a purine transport system were performed in June and July, 1983. The results of these studies have been presented at the University of California, Irvine, July 26, 1983, at a Review of Air Force Sponsored Research.

105. Theory:

Two different types of experiments were performed to show the potential effects of the reaction of PFDA, insulin, or purines (hypoxanthine, uric acid, adenine, and xanthine) on the transporter for 2-aminopurine (AP) in the membrane of L5178Y cells. The first type of experiment was designed to show whether the reactants could produce a rapid and direct influence on the function of the carrier. The second type of experiment was designed to show a potential slow and indirect effect on the carrier of (AP).

1051. Direct Effects on the Transport System for 2-Aminopurine.

In a typical experiment of this type, living cells are incubated with a nonmetabolizable substrate at a temperature sufficient to permit substrate influx through the cell membrane but below the optimal temperature for cell growth; a temperature of 30° is usually appropriate. After the substrate is preloaded to the equilibrium concentration, the cells are cooled to 4° and sedimented in a refrigerated centrifuge. The preloaded cells are resuspended and centrifuged twice in cold (4°) buffer to remove excess substrate and the fluorescent compounds in the growth medium. The preloaded cells are then resuspended in a warm buffer that contains a known concentration of stimulant or inhibitor substance. The efflux rate is then determined immediately by a filtration-flow-fluorescence procedure (see section 106).

The phenomenon of stimulation of substrate flux with a compatible substrate on the opposite side of the membrane is usually called "accelerated exchange diffusion" (AED). A more accurate title for this phenomenon may be "trans accelerative exchange flux." The rate limiting reaction of one-way facilitated substrate flux is usually the recovery reaction of the unloaded carrier. The substrate-loaded carrier recovers faster than the unloaded carrier. Since the carrier reaction is ordinarily accelerated by the presence of a substrate in the trans position, exchange reactions are usually faster than the zero-trans (one-way flux) reaction. An analysis of the mechanism of AED and an appropriate rate equation can be found in Wigler (1981). Unfortunately, data from AED experiments are difficult to interpret; additional references on this phenomenon have been provided (see Wigler, 1981).

The phenomenon of inhibition of substrate flux with an inhibitor on the opposite (trans) side of the cell membrane is called trans efflux inhibition (TEI, Wigler, 1981). The first mechanistic analysis to give the derivation of a steady state rate equation for initial velocity was reported by Wigler in

1981. Although the TEI phenomenon was known for a long time, this phenomenon could not be interpreted without a rate equation. The mechanism and rate equation are quite simple, and indicate that it is possible to estimate the rate constant for recovery (g) of the unloaded carrier. It is necessary to determine the initial velocity of substrate efflux at several different trans inhibitor concentrations.

The experiments presented in sections 106 and 107 indicate that PFDA has no direct effect on the efflux of AP. Thus, the PFDA-carrier interaction cannot be analyzed by the AED or TEI kinetic treatments. This observation serves as a control in understanding the indirect effects of PFDA on the purine carrier (see section 107).

Preliminary data (section 107) indicate that the efflux of AP is inhibited by uric acid (UA) in the external buffer. After this experiment has been repeated with different concentrations of UA it may be possible to analyze the inhibition reaction with the following rate equation:

$$v = \frac{V_S}{1 + \frac{[I]}{g K_I} + \frac{K_S}{[S_i]}} \quad (1)$$

In the foregoing steady state rate equation (Wigler, 1981), v is the initial velocity for AP efflux, V_S is the maximum velocity, V'_S is the turnover number, $[I]$ is the concentration of UA in the external buffer, g is the rate constant for recovery of the unloaded carrier, K_I is the inhibition constant (dissociation constant of the UA-carrier complex), K_S is the Michaelis constant, and $[S_i]$ is the intracellular concentration of AP. If v is determined at different $[I]$ values, it will be possible to determine the parameters g and K_I ; the g value for purine transport has not been reported.

1052: Indirect Effects on the Transport System for 2-Aminopurine.

In this type of experiment, the L5178Y cells are given a prior incubation with a potential inhibitor (PFDA) dissolved in the complete growth medium for 24 hr. The "damaged" cells are then isolated by centrifugation and most of the medium is removed by aspiration. The cells are then preloaded with the substrate as described in section 1051. The excess substrate and medium is removed with cold buffer and the rate of efflux is determined by the filtration-flow fluorescence procedure.

The kinetic treatment of indirect inhibition experiments utilizes the zero-trans reaction (Hankin et al., 1972). The derivation of the zero-trans rate equation is based on the mechanism in Fig. 1. The mechanism suggests that the unloaded carrier can undergo a conformational change with the active site open to the cytoplasm of the cell (inside open) or the carrier site can be in the "outside open" conformation. A molecule of intracellular substrate reacts with the "inside open" carrier to form a carrier-substrate complex. The complex undergoes a conformational change to expose the active site to the external medium and substrate is released outside of the cell. In the last reaction, the unloaded carrier changes from the "outside open" structure to "inside open"; this reaction is called carrier recovery and can be identified by the rate constant, parameter g.

A rate equation was derived from the mechanism in Fig. 1. The rate equation was rearranged in the form of the Michaelis-Menten equation (see equation 2); the Michaelis constant and V_S are given in equations 3 and 4. The rate constants for the forward reaction of Fig. 1 are represented by a, c, e, and g, and the rate constants for the reverse reaction are b, d, and h. The concentration of carriers is $[C_t]$. The rate constants for K_S and V_S are the same as in the case of a TEI experiment but different from an AED experiment (see Wigler, 1981).

$$v = \frac{V_S}{1 + K_S/[S_i]} \quad (2)$$

$$K_S = \frac{(1 + h/g) (bd + be + ce)}{a (c + d + e + ce/g)} \quad (3)$$

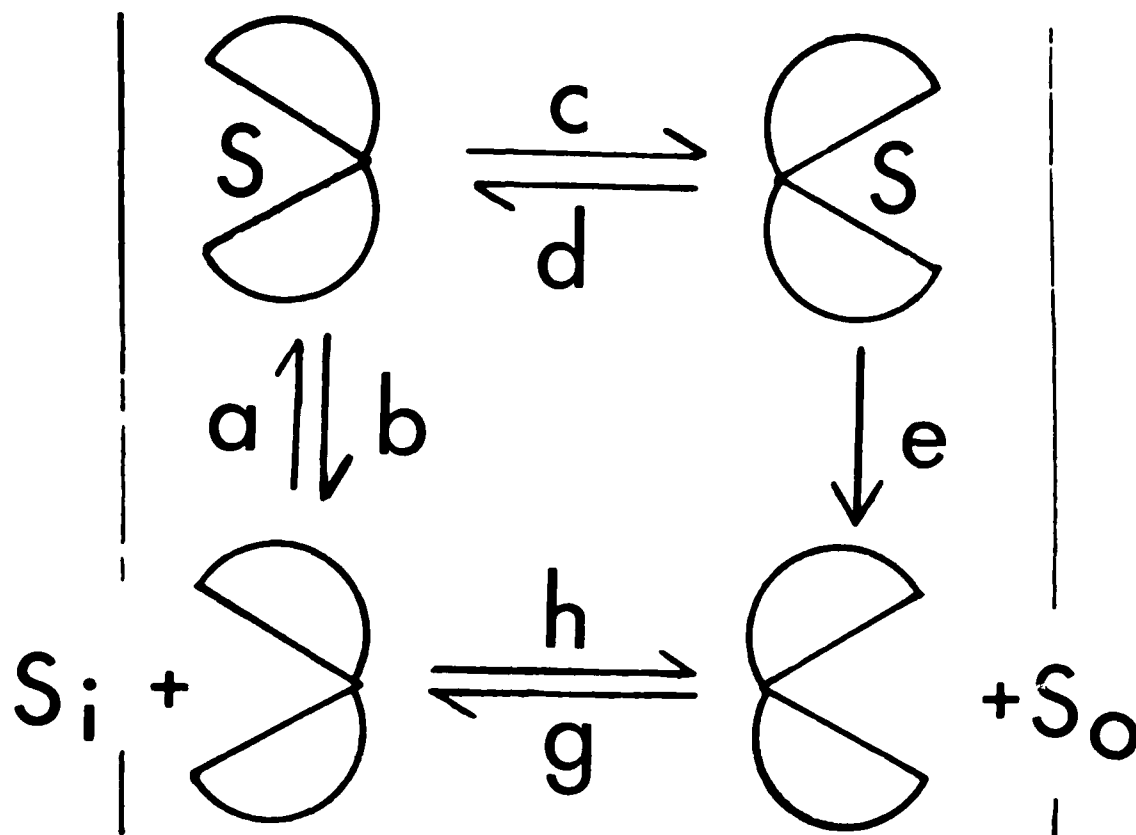


Fig. 1: A model that shows the conformation change of the purine carrier and the zero-trans reaction for purine efflux. The purine substrate inside the cell is indicated by S_i and the external substrate is indicated by S_o . The rate constants for the forward reaction are a , c , e , and g ; the reverse constants are b , d , and h .

$$V_S = \frac{ce [C_t]}{c + d + e + ce/g} \quad (4)$$

Equations 3 and 4 indicate three rate constants appear in the expression for K_S (constants a, b, and h) that do not appear in the expression for V_S . An inspection of Fig. 1 shows that the rate constant a, b, and h are involved with the binding of S_i to the unloaded "inside open" carrier or with the conformational change of the "inside open" to the "outside open" structure. Apparently, these three parameters have no effect on the V_S value. The V_S and K_S of L5178Y cells, given a prior incubation with PFDA, are compared with the same parameters for untreated control cells. If the PFDA treatment changes the reactivity of the unloaded "inside open" carrier (but has no effect on the carrier-substrate complexes), the K_S of PFDA treated cells will be different from the K_S for controls. The V_S may be the same for the PFDA-treated cells and the controls.

Preliminary experiments indicate that a prior incubation of L5178Y cells with 200 $\mu\text{g/ml}$ PFDA at 30° for 24 hours gives a total inhibition of AP efflux. These experiments (see section 106) will be repeated with several different AP concentrations to determine the effects of PFDA on K_S and V_S of the AP efflux reaction.

106. Experimental:

The experiments to show the potential direct (rapid) effects of the reactants PFDA, insulin, or purines (hypoxanthine, uric acid, xanthine, and adenine) will be described separately from the experiments that show an indirect (delayed) effect.

1061: Direct Effects on a Purine Transport System.

The PFDA was obtained from Aldrich Chemical Co. and the other reagents were obtained from Sigma Chemical Co. In these experiments a stock solution of PFDA in ethanol was prepared and diluted into the buffer; the final concentration of ethanol was 0.01%. In recent experiments we have observed that the sodium salt of PFDA is soluble in aqueous solution. This observation may eliminate the need for DMSO (see Andersen *et al.*, 1982) or ethanol in future experiments.

An inoculum of 100 L5178Y cells (gift of Dr. C.F. Arlett, Cell Mutation Unit, Brighton, England) was added to 9.0 ml McCoy's 5A medium plus 1.0 ml horse serum (10FM. Andersen *et al.*, 1982). The suspension was incubated at 37° until the cell count was 2×10^5 cells/ml (approximately five days). The medium contained 100 IU/ml penicillin and 100 µg/ml streptomycin. The cells were centrifuged at 3000 RPM for 10 min and 9.0 ml of the supernatant was removed by pipet. A solution of 2-aminopurine (AP) in medium was added to a final concentration of 100 µM AP and the 1.0 ml suspension was incubated with mild agitation for 2 hr at 30°.

The cell suspension was cooled to 4°, centrifuged at 3000 RPM for 10 min, and the supernatant was discarded. The cell pellet was resuspended in cold phosphate-buffered saline, pH 7.4 (PBS, 4°) twice and centrifuged twice to remove excess substrate. The preloaded cell pellet was resuspended in warm (21°) PBS buffer that contained different reagents (PFDA, hypoxanthine, uric acid, bovine insulin, adenine, or xanthine). The 5.0 ml cell suspension was quickly poured into a filtration-flow apparatus with a 5 micron Millipore filter to remove the cells. The supernatant was drained into a quartz flow cell; the flow rate was regulated at 1.0 ml per min by a Buchler Polystaltic Pump downstream from the flow cell. Ultraviolet light at 308 or 312.5 nm from a Hg-Xe lamp was used for excitation of the AP in the solution, and the emission of AP was estimated at 370 nm with an SLM-Aminco 8225 Photon-Counting Fluorescence Spectrophotometer and a PT 9635QA Photomultiplier Tube. The light emission was determined at 4 sec intervals and the initial velocity of efflux was determined from an apparent zero order plot up to 32 sec. The volume of the cell suspension decreased 0.5 ml during the determination of the initial rate; a sketch of the flow-filtration fluorescence apparatus is given in section 107.

1062: Indirect Effects of PFDA on a Purine Transport System.

The L5178Y cells were grown in McCoy's 5A plus 10% horse serum as in section 1061. Suspensions of 37×10^6 cells in 10 ml medium that contains 10% serum were prepared with the following treatment conditions: a) 100 or 200 µg/ml PFDA final concentrations; b) 50 or 100 mU/ml bovine insulin final concentrations; c) PFDA at 100 or 200 µg/ml plus insulin at 50 or 100 mU/ml; and d) no additions (controls). The different treatment conditions

are listed in Table 1. The foregoing suspensions were given a prior incubation with mild agitation for 24 hr at 30° and the cells were subsequently counted in each suspension, with a Neubauer Hemacytometer. The cells were centrifuged at 3000 RPM for 10 min and 9.0 ml of the supernatant was removed by pipet. A solution of AP was added to a final substrate concentration of 100 μ M and the 10 ml suspension was incubated for 2 hr at 30°. The suspension was cooled to 4°, centrifuged at 4°, and the supernatant was discarded. The pellet was resuspended and centrifuged in cold PBS as in section 1061. The preloaded cells were resuspended in warm PBS and the 5.0 ml cell suspension was poured into the filtration-flow fluorescence apparatus to estimate the initial rate of AP efflux.

The emission values were converted to the concentration of AP from a plot of emission intensity versus AP concentration from 0.02 to 0.32 μ M, performed each day. The data were also corrected to a cell count of 10^{12} cells per liter (10^9 cells/ml). Prior incubations of L5178Y cells were also performed at 37° with 100 μ g/ml PFDA and 100 mU/ml insulin; the experimental conditions were similar to the 30° treatment.

107. Results:

Experiments were conducted to determine the initial rate of AP efflux from L5178Y cells, using an aliquot and quench assay procedure. At best, individual time points could be taken at 8 sec intervals and the high pipetting error interfered with the velocity determination.

Determinations of the initial velocity of membrane transport require a high speed kinetic procedure (Wohlhueter et al., 1976). A prototype filtration-flow apparatus was constructed by the Principal Investigator from scrap metal and routine laboratory parts; a sketch of the apparatus is shown in Fig. 2. An assembly that consists of a hypodermic barrel and a membrane filter holder is mounted on top of the cell compartment lid of the fluorescence spectrophotometer with a tripod. The filter effluent drains into a quartz fluorescence flow cell through medical plastic tubing. The outflow plastic tubing from the quartz cell passes through a pump to regulate the rate of flow. Excitation light illuminates the flow cell from behind, and the emission light is detected at a rectangle with a high sensitivity photomultiplier tube. Although it is possible to measure the emission light at 2 sec intervals, the SLM instrument is not able to store the kinetic data at that speed. On the other hand, the SLM 8225 electronic package is able to measure and store emission data at 4 sec intervals.

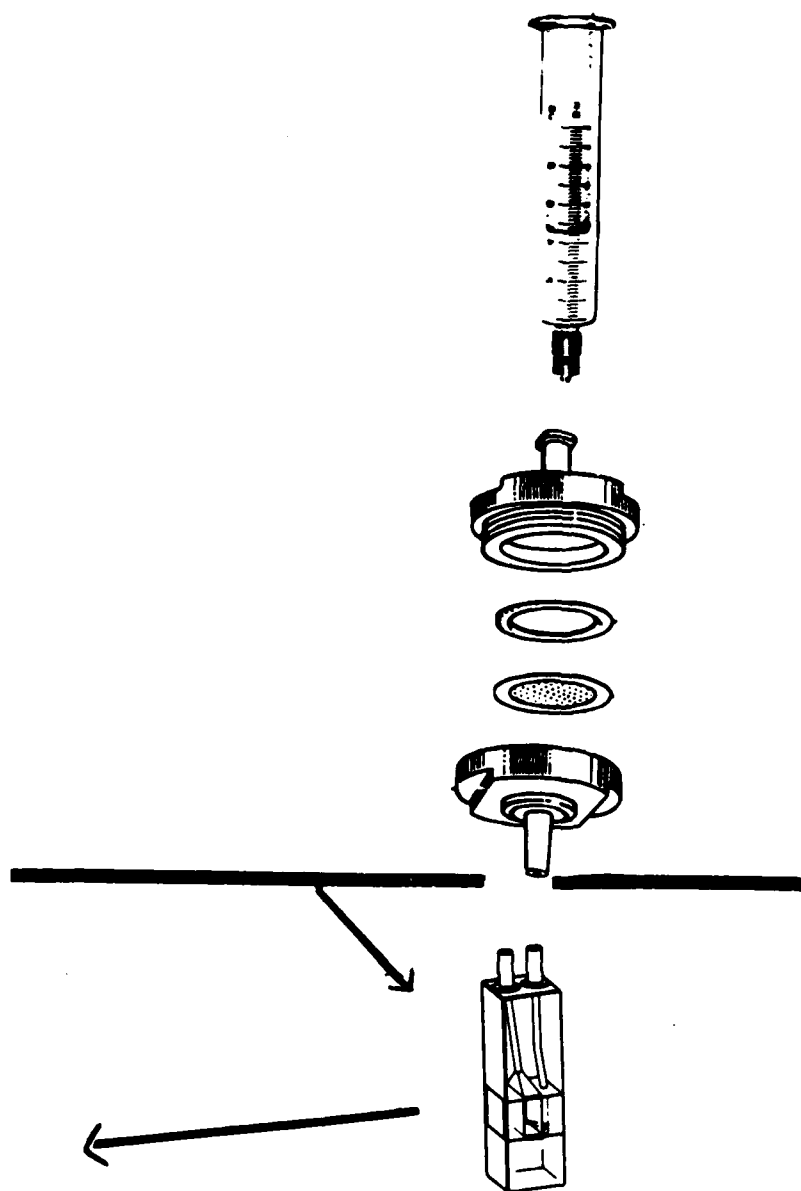


Fig. 2: A sketch of the flow-filtration apparatus for the rapid determination of the rate of 2-aminopurine efflux. The suspension of preloaded cells is placed in a syringe barrel connected to a holder for a 5.0 micron membrane filter. The efflux buffer is drained through a quartz flow cell with medical plastic tubing. The excitation light enters the cell from the rear and the emission light is detected at a right angle.

Certain problems were noted in the operation of the prototype flow apparatus. There is a need for precise measurement and regulation of the temperature of the cell suspension in the syringe barrel. In addition, there may be a need for a constant-volume siphon for the cell suspension (see section 109). There is an inherent advantage to flux measurements from a small volume into a large volume in the direction of efflux (Miller, 1975). Furthermore, an assay based on fluorescence can detect substrate molecules at very low concentrations. Thus, the assay for membrane transport described in this report has the potential of becoming the best method of estimation of substrate flux ever developed.

L5178Y cells preloaded with AP were mixed with a solution of hypoxanthine (at a final concentration of 500 μM) in PBS buffer at 21⁰. The resultant suspension was quickly poured into the flow-filtration apparatus and the effluent buffer was excited with light at 312.5 nm (a wavelength of high emission for the mercury lamp). The emission data was corrected for a cell count of 10^{12} cells per liter, based on counts of each cell suspension. The effect of time on the increase in emission at 370 nm (the peak for AP) is shown in Fig. 3. The experiment was repeated with the addition of 500 μM of uric acid, adenine, xanthine or with no addition (control). It can be seen from Fig. 3 that efflux of AP is stimulated by hypoxanthine and inhibited by uric acid in the external buffer. The addition of adenine or xanthine at 500 μM had no significant effect on the rate of AP efflux in comparison with the control. Guanine and isoguanine could not be tested with this system because these compounds are insoluble at the 500 μM concentration levels in aqueous buffer at pH 7.4. Insulin had no direct effect on efflux.

In a separate experiment, PFDA was tested as a potential trans (direct) inhibitor of AP efflux; the results are shown in Fig. 4. The cell pellet was resuspended in a PFDA solution in PBS at 21⁰ and the suspension was poured into the flow-filtration apparatus. The results of Fig. 4 indicate that PFDA at 100 $\mu\text{g/ml}$ or 200 $\mu\text{g/ml}$ in the external buffer has no direct effect on AP efflux in comparison with the controls (no additions). The experiment of Fig. 4 is the same as the experiment of Fig. 3 except that excitation of the effluent was at 308 nm, the peak excitation wavelength for AP. (The emission from a standard solution of AP at the Hg emission of 312.5 nm excitation was elevated 25% in comparison with excitation at 308 nm).

L5178Y cells were given a prior incubation with 100 $\mu\text{g/ml}$ PFDA in medium

plus serum for 24 hr at 30⁰. The cells were subsequently preloaded with AP to a final concentration of 100 μ M and the cells were rinsed in cold buffer to remove excess substrate. The cells were resuspended in warm buffer and the suspension was quickly poured into the flow device. The data of Fig. 4 show that the efflux of AP from these PFDA-treated cells is strongly inhibited.

An experiment was performed to determine the effect of prior incubation with 100 μ g/ml PFDA in medium plus serum for 24 hr at 37⁰. (PFDA at 200 μ g/ml could not be tested at this temperature because most of the cells are lysed after 24 hr.) As shown in Fig. 5, efflux of AP by PFDA-treated cells is slower than efflux by control cells. Another goal of the foregoing experiment was to determine whether a combination treatment of PFDA plus insulin during the 24 hour prior incubation would protect the cells from the effects of PFDA alone. The data of Fig. 5 indicate that insulin protects the cells from PFDA; the AP efflux rate from the PFDA plus insulin treated cells is higher than for the cells treated with PFDA alone. It was also observed that a prior incubation of L5178Y cells with insulin alone (at 37⁰ for 24 hr) produces a partial inhibition of AP efflux.

In a subsequent experiment, all the cells received a prior incubation in medium plus serum for 24 hr at 30⁰. Additions of PFDA and insulin were used in different combinations as summarized in Table I. The cell count of the control suspensions increased from 3.7×10^6 cells/ml to 4.4×10^6 cells/ml in 24 hr at 30⁰. (Cell counts were estimated with a Hemacytometer). As shown in Table I, PFDA at two different concentrations produces a stable cell count in comparison with the count at zero time. The addition of insulin, however, may produce a small stimulation of cell division. The effect of combination treatments of insulin plus PFDA usually produces a level of cell division that is intermediate compared to the use of either compound alone. The L5178Y cells were also tested for a capability to exclude Trypan Blue; the exclusion test is used in Table I as a criterion of cell viability. Apparently, the treatment of cells with PFDA for 24 hr at 30⁰ does not produce a significant increase in nonviable cells.

The cells described in Table I were preloaded with AP to a concentration of 100 μ M and excess substrate was removed with two cold rinses. The results are summarized in Figs. 6 and 7. It should also be noted that the data of Figs. 6 and 7 were converted from emission to the concentration of AP with a standard curve. (An excitation of 312.5 nm was used in this experiment). Although the prior incubation with 200 μ g/ml PFDA at 30⁰ produces a total inhibition of AP efflux, the addition of 50 mU/ml insulin provides a substantial degree of

Table I: Effect of PFDA and Insulin in Medium on the Count of L5178Y Cells. The Cells, in a 10 ml suspension, were treated for 24 hr at 30° and the cells were then tested for exclusion of Trypan Blue. Controls were counted in duplicate; at zero time the count was 37×10^5 cells/ml.

		*	
PFDA	Insulin	Cell Count	
$\mu\text{g/ml}$	mU/ml	Viable	NonViable
NONE	NONE	44(44)	7(5)
100	NONE	36	4
200	NONE	34	3
100	50	40	2
200	50	28	3
100	100	38	6
200	100	32	3
NONE	50	54	4
NONE	100	44	4

*Cells/ml ($\times 10^{-5}$)

protection (see Fig. 6). The results with 100 $\mu\text{g/ml}$ PFDA are consistent with the foregoing observation; however, it is difficult to predict whether the decrease of the initial velocity is statistically significant. The results of Fig. 6 show that prior incubation with 50 mU/ml insulin produces a slight stimulation of AP efflux as well as stabilization of the AP efflux capability of the L5178Y cells (when in combination with PFDA).

The results of Fig. 7 show the effect of 100 mU/ml insulin in combination with PFDA. In this case, the higher insulin level is less effective in protecting the AP carrier system from PFDA than 50 mU/ml insulin. These findings show that PFDA has a very significant effect on a carrier for purine transport in the membrane of L5178Y cells. The PFDA effect is partially reversed by insulin.

108. Discussion:

The results of Fig. 3 show that hypoxanthine in the external buffer may increase the initial rate of AP efflux. This finding suggests that these two purine substrates share the same carrier in the cell membrane of L5178Y (Wigler, 1981). Since adenine and xanthine do not influence the rate of AP efflux, the latter purines may be transported by a different carrier protein of different specificity. Although AP and hypoxanthine are structurally related to guanine, it is difficult to determine whether these purines share the same carrier due to solubility problems with guanine. Further experiments with different concentrations of AP and hypoxanthine are indicated here, to establish the carrier mechanism.

The trans inhibitory effect of uric acid (Fig. 3) suggests that the latter purine binds to the AP carrier, but is not an efficient substrate for influx. This finding should be verified by further experiments with different uric acid concentrations. In addition, experiments shall be performed with other potential transport inhibitors, such as colchicine.

The primary metabolic site of action of PFDA is unknown at the present time. All available evidence suggests that inhibition of an enzyme in the biosynthesis of saturated lipids is the primary site. Alterations in the lipid composition and the fluidity of the cell membrane would produce significant secondary metabolic changes. If the primary site of PFDA action is lipid synthesis, a direct inhibition of the purine carrier by PFDA would not be expected. When efflux of AP from L5178Y cells was estimated with PFDA in the external buffer, the initial rate observed was the same as the controls

within the experimental error. (see Fig. 4). On the other hand, the prior incubation of L5178Y cells with PFDA for 24 hr. produces an inhibition of AP efflux. These observations are consistent with the concept that changes in membrane lipids increase the levels of inactive purine carrier.

In the experiments from this laboratory, most of the L5178Y cells do not survive a 24 hr. treatment with 200 $\mu\text{g/ml}$ PFDA at 37° . It seems reasonable to determine whether the increase in inactive purine carrier represents an early stage in cell destruction. Cell counts were performed prior to the efflux experiment in Fig. 5; the data is shown in Table II. The results of Table II show that L5178Y cells undergo cell division in 100 $\mu\text{g/ml}$ PFDA at 37° for 24 hr. but at a slower rate than the control cells. A normal rate of cell division is restored when insulin is added to the suspension in combination with the PFDA. The AP efflux of PFDA treated cells, that received a prior incubation at 37° , is inhibited in comparison with the controls (see Fig. 5). These observations suggest that an increased level of inactive carrier is an early event in the destruction of leukocytes by PFDA.

The potential effects of environmental agents on receptors in the cell membrane has been discussed by Cook et al., (1976). One possible mechanism for the inhibition of AP efflux prior to cell death, is a modification in the turnover of the carrier protein. PFDA may be an inhibitor of carrier protein synthesis in the cell membrane. Another hypothesis suggests that the composition of lipids surrounding the carrier would influence transport activity. This is an attractive idea because of the other known effects of PFDA treatment. In this mechanism, the presence of an excessive level of unsaturated phospholipids would produce an inactive purine carrier. A generalized hypothesis that describes the effect of PFDA on the AP carrier is shown in Fig. 8. It is suggested that the AP purine carrier may exist in an active or an inactive form. The activity level may be controlled through inhibition of protein synthesis or the interaction between the carrier and the membrane phospholipids. It is suggested in Fig. 8, that PFDA inhibits the conversion of the inactive carrier to the active carrier.

The physiological function of the purine carrier is to facilitate the excretion of the purine end products of catabolism. If the concentration of these compounds increases because the efflux carrier has been damaged by a toxicant, the cellular metabolism is subject to feed back inhibition

Table II: Effect of PFDA and Insulin on the Count of L5178Y cells. The cells, in a 44 ml suspension, were incubated for 24 hr at 37⁰ in medium and the cells were counted; at zero time the count was 10⁶ cells/ml.

PFDA	Insulin	Cell Count
$\mu\text{g/ml}$	mU/ml	Cells/ml ($\times 10^6$)
NONE	NONE	2.1
100	NONE	1.7
100	100	2.1
NONE	100	1.8

(Wigler, 1978). Uric acid, an end product of purine catabolism, is toxic to cells at elevated concentrations. Damage to the purine carrier for efflux may be one significant reason for the loss of cell viability due to PFDA treatment.

It is of interest to compare the effects of prior incubation of L5178Y cells with PFDA at 37⁰ with the results of a similar experiment at 30⁰. When a concentration of 100 $\mu\text{g/ml}$ PFDA is used for 24 hr the effect on AP efflux is about the same at the two different temperatures. In the experiments performed in this laboratory, however, it was impossible to investigate transport with higher PFDA concentrations at 37⁰ because most of the cells are lysed in the presence of 200 $\mu\text{g/ml}$ of PFDA. Thus, a total inhibition of AP carrier activity could not be estimated at 37⁰. Inhibition of AP efflux could be reproduced with a range of PFDA concentrations at 30⁰, however, with minimal morphological effect on the L5178Y cells (Machado, 1983).

The results of Fig. 6 indicate the presence of an insulin receptor in L5178Y cells. The physiological effects of insulin-receptor interaction have been extensively studied in the last three years (Seals and Czech, 1981). Apparently, insulin binding to the receptor at the membrane surface leads to the release of peptide mediators. These mediators may stimulate or repress intracellular enzymes or carriers in the membrane. The results of Fig. 6 show a slight stimulation of AP efflux after a prior

incubation with insulin. The data is not sufficient at the present time, to determine whether this is a statistically significant observation.

A 24 hr treatment with 200 $\mu\text{g/ml}$ PFDA at 30° produces a total inhibition of the AP efflux reaction. The combination treatment of 50 mU/ml insulin plus 200 $\mu\text{g/ml}$ PFDA preserves approximately 60% of the AP transport capacity of the L5178Y cells. One possible explanation of this observation is that an insulin mediator could stimulate the synthesis and deposition of saturated lipids in the cell membrane to restore the unsaturated/saturated ratio and the membrane fluidity to normal physiological levels. Another possibility is that an insulin mediator may inhibit the synthesis of unsaturated fatty acids.

The scheme of Fig. 8 shows the opposing effects of insulin and PFDA on the turnover of inactive and active AP carrier. It is suggested an insulin mediator forms a complex with the inactive carrier that is subsequently converted to the active carrier. The mediator-carrier complex is relatively resistant to the action of PFDA.

Many aspects of this scheme can be tested by direct experiment. In addition to further experiments on AP efflux, it will be important to determine the effects of PFDA and insulin on the lipid and protein composition of L5178Y cells.

109. Comparative Applications:

The purpose of this section is to discuss the experiments planned for the next two months and to consider the priorities for the choice of subsequent comparative experiments. In brief, we have developed the apparatus and methodology for a high speed kinetic assay of purine efflux, based on fluorescence. Most of the transport assays in current use depend on isotope labeled substrates. Isotope counts are based on levels of radioactive atoms; however, the cpm may not be proportional to the substrate concentration. Uziel and Selkirk (1979) have suggested that the specific radioactivity of extracellular uridine must be known to correlate cpm with the concentration of uridine excreted from growing cells. Fluorescence has the advantage that emission from a fluorescent substrate is directly proportional to the substrate concentration. In view of the high speed and sensitivity of this method, we may have the best known transport assay system. (see Eidelman and Cabantchik, 1983).

The prototype flow-filtration apparatus was functional on the first

attempt; the experiment in Fig. 5 was the first trial run of the apparatus. Furthermore, in the same experiment it was possible to demonstrate inhibition of AP carrier by a prior incubation with PFDA. The protective effect of insulin was also demonstrated. Treatment with 100 $\mu\text{g/ml}$ PFDA for 24 hr at 37° , has a relatively small effect on cell division (Table II) and an insignificant effect on cell morphology (Unpublished Electron Micrographs from the laboratory of Prof. E.A. Machado, M.D., Memorial Research Center).

One possible explanation for the inhibition of AP efflux by the prior incubation with PFDA is an inability of the treated L5178Y cells to preload with the fluorescent substrate. The experiments in this report were performed before July 31, 1983; however, we have determined the effect of a 24 hr PFDA-treatment on AP preloading in a more recent experiment. The L5178Y cells were treated with 200 $\mu\text{g/ml}$ PFDA for 24 hr at 30° and the cells were subsequently preloaded with 100 μM AP for two hr at 30° . The suspension was cooled to 4° and the cell pellet was rinsed twice with cold PBS. The cells were disrupted with a glass homogenizer and the resultant suspension was mixed with PBS. Insoluble cell debris was sedimented by centrifugation; the supernatant was analyzed for soluble fluorescent compounds with excitation by light at 312.5 nm and emission at 370 nm. The intensity of emission of the solute from PFDA-treated cells is approximately the same as the emission of the controls. Thus, the PFDA-treated cells are preloaded with AP to the same level as the controls.

The prototype flow device will be modified to include a jacket for circulation of temperature regulated solvent (an ethylene glycol water mixture) around the syringe barrel. The probe of a thermister thermometer will be attached to the filter-holder for a continuous determination of the temperature of the cell suspension. The volume of the cell suspension will be increased from 5 ml to 10 ml. Since the volume of the cell suspension decreases by 0.5 ml during the 32 sec rate determination, the volume change will be 0.5%. If this procedure proves inadequate, a constant volume siphon will be constructed.

The first objective in the second year of this project is to characterize the AP carrier. The L5178Y cells (controls) will be preloaded with different concentrations of AP from 100 μM to 500 μM and the rate of efflux will be determined. The data will be plotted by a linear form of the Michaelis-Menten equation and the Michaelis constant and maximum velocity will be determined. In another experiment, the cells will be preloaded with 100 μM

AP and the rate of efflux will be determined with different concentrations of hypoxanthine in the external buffer from 300 μM to 1500 μM . The apparent K_S and V_S for accelerated exchange diffusion with hypoxanthine will be determined.

The trans inhibitor, uric acid, will be tested at different concentrations in the external buffer. The data will be used to determine the inhibition constant for uric acid. Another important question is whether the AP efflux depends on the ATP concentration, or the sodium ion gradient. The ATP content of the cells can be modified by changing the level of nutrients in growth medium or with a metabolic inhibitor. The sodium gradient can be changed by placing the cells in a buffer that contains 0.14 M KCl instead of NaCl.

The results of Fig. 6 and Table I indicate that we can prepare L5178Y cells with a total deficiency in AP transport, but a "normal" capacity for cell division. It will be interesting to determine whether the damage to the turnover system of the carrier can be retained in the cell line. The L5178Y cells will be given a prior incubation with 200 $\mu\text{g/ml}$ PFDA in complete medium for 24 hr at 30°. The PFDA plus medium will be removed and the cells will be incubated for 24 hr in McCoy's medium plus horse serum (no PFDA) for 24 hr. The cells will be preloaded with AP and the rate of AP efflux will be determined. If the purine efflux system does not recover within 24 hr we shall try to grow the cells for one week (the count at 37° of control cells doubles in 12 hr), to determine if there has been a gene defect due to PFDA. Insulin will be added to selected cell suspensions during the period of recovery from PFDA, to find out whether insulin can enhance the recovery.

In a separate experiment, L5178Y cells will be given a prior incubation with 50 mU/ml insulin for 24 hr at 30° and subsequently treated with PFDA. The cells will then be preloaded with AP and purine efflux will be assayed. It should be of interest to find out whether a prior treatment with insulin can protect the cells against PFDA.

A study of the morphology of L5178Y cells treated with 200 $\mu\text{g/ml}$ PFDA for 24 hr at 30° will be performed. The electron micrographs will be prepared in the laboratory of Prof. E.A. Machado, (Dr. Machado has observed that cells treated with 100 $\mu\text{g/ml}$ for 24 hr at 37° have the same structural features as the controls).

After the foregoing experiments are completed, we should have enough data to submit a manuscript for publication.

There are four potential objectives that could follow the results obtained on the PFDA-purine transport interaction. These are listed as follows:

- a) The effects of prior incubation with PFDA on nucleoside efflux;
- b) The effects of PFDA on the lipid and amino acid composition of L5178Y cells (If these studies are in progress at Wright-Patterson AFB, we shall avoid duplication of effort);
- c) The effects of insulin on the lipid and amino acid composition of the PFDA treated and control cells;
- d) The effects of prior incubation with 2,3,7,8,-tetrachlorodibenzo-p-dioxin (TCDD) on the efflux of 2-aminopurine from L5178Y cells.

At the present time, I am uncertain which of the foregoing goals would be most valuable in furthering the research objectives of the Air Force. I believe that priority levels for these goals should be established in consultation with the Program Manager of this project.

We have an unusual opportunity to determine whether prior incubation with PFDA will inhibit the efflux of a nonmetabolized fluorescent nucleoside. Dr. J.C. Martin of Syntex, Inc. has given us a small sample of 9-(1,3-dihydroxy-2-propoxymethyl)-2-aminopurine (acycloribofuranosyl-2-aminopurine). This sample is sufficient for one preliminary experiment to show whether prior incubation with PFDA can inhibit the efflux of a fluorescent nucleoside. We have already demonstrated cellular efflux of acycloguanosine (Acyclovir, Antiherpes drug from Burroughs-Wellcome, Inc.), but the nucleoside analog is fluorescent only at extremely low pH. Thus, the data with acycloguanosine was not reproducible. [The acycloribofuranosyl-2-aminopurine is structurally the same as acycloguanosine with the 6-oxy group replaced by hydrogen atoms.] The Syntex compound would be just as fluorescent on a molar basis as 2-aminopurine (AP).

Another potential fluorescent nucleoside substrate is 5'-chloro-5'-deoxyformycin. I believe that we could prepare this analog in this laboratory within one month. The chloro derivative should be resistant to purine nucleoside phosphorylase and adenosine kinase. Thus, the formycin derivative may not be metabolized during the two hr preloading of L5178Y cells at 30°.

The second potential goal is to incubate L5178Y cells with PFDA as in Table I, isolate the cells, and determine the fatty acid composition by gas chromatography. The equipment for chromatography of fatty acids is available in the laboratory of Dr. Davis Lin of the Memorial Research Center. In addition, he has an

apparatus for the analysis of amino acids that would allow us to determine whether protein synthesis is modified by PFDA treatment.

Dr. M.E. Andersen (Toxicology Branch, AFAMRL/THT, Wright-Patterson AFB) has indicated that he can supply a small sample of TCDD for tests with the L5178Y cells. The Principal Investigator would provide transportation for the TCDD sample. It may be important to determine whether TCDD interferes with the carrier for 2-aminopurine. The studies by Dr. Andersen and Col. C.T. Olson suggest that the effects of TCDD would be similar to the effects of PFDA.

110. References and Published Abstracts:

The reference list in this report is not intended to be comprehensive. The fluorescence efflux technique has been used by Eidelman and Cabantchik (1983). The Principal Investigator will undertake a complete literature survey in time for the report on AFOSR 82-0261B in March, 1984.

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- 16) P.W. Wigler and Shah, Y.B. (1983 Abstract) "Toxicant Effects on the Efflux Rate of Fluorescent Substrates from L5178Y Cells" 35th Southeast Regional Meeting, American Chemical Society, Charlotte, N.C., November 11, 1983, in press.

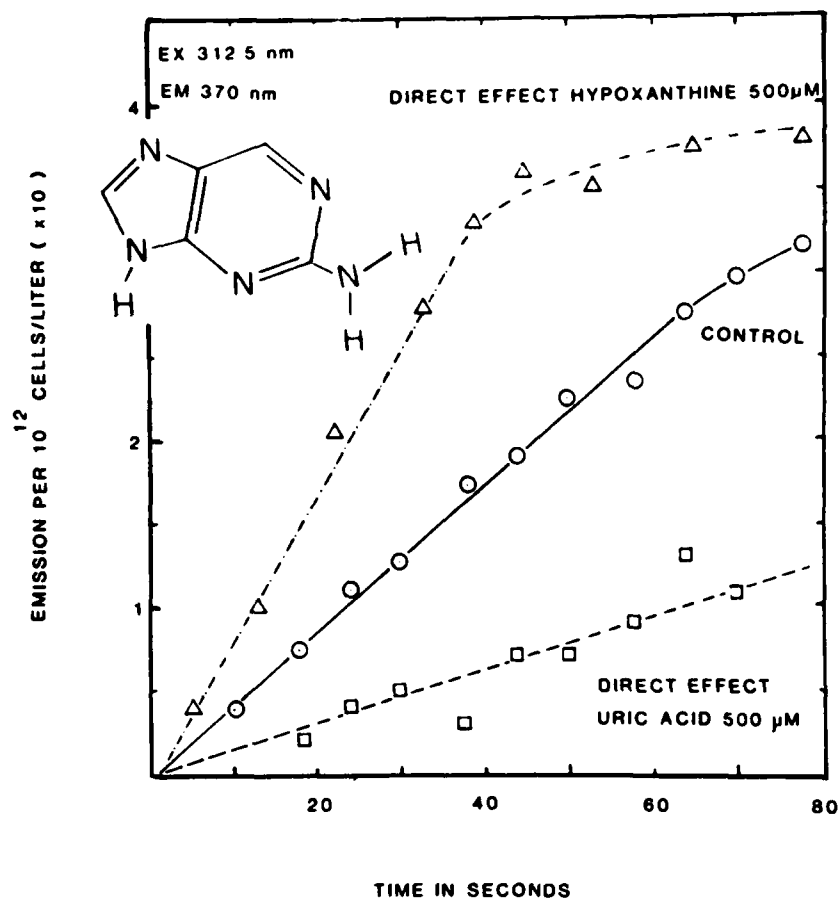


Fig. 3. Direct Effects of Hypoxanthine and Uric Acid on Efflux of 2-Aminopurine from L5178Y Cells. The cells were preloaded at 30° with 100 μM AP and rinsed with cold buffer. The cells were mixed with PBS that contained 500 μM hypoxanthine (Δ), 500 μM uric acid (◻), or no addition (◉). The 21° suspension was poured through a 5 micron filter and drained through a quartz cell. The excitation wavelength was 312.5 and emission was measured at 370 nm (the AP maximum). Data were adjusted for 10^{12} cells per liter.

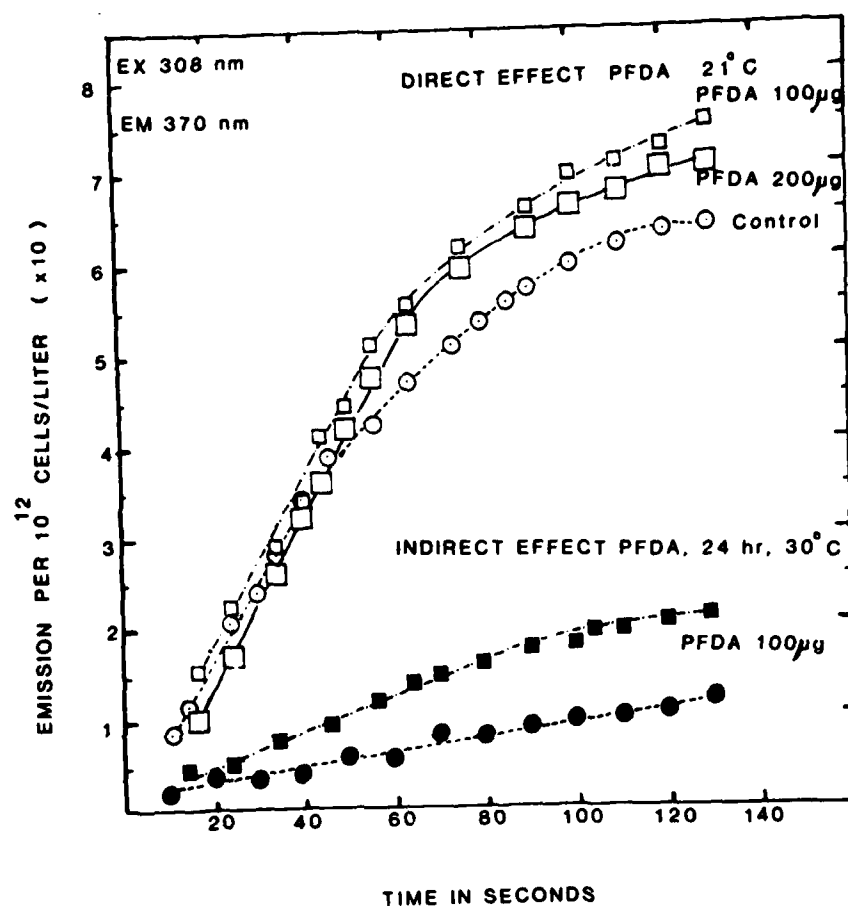


Fig. 4. Direct and Indirect Effects of PFDA on Efflux of AP from L5178Y Cells. Cells were preloaded as in Fig. 3 and resuspended in 100 µg/ml (□), 200 µg/ml (◻) PFDA, or no addition (○). The cell suspension was filtered, the excitation wavelength was 308 nm, and emission was measured at 370 nm. The indirect effect was demonstrated by a prior incubation of L5178Y cells with 100 µg/ml PFDA (■), 200 µg/ml PFDA (●), for 24 hr at 30°. The cells were then preloaded with 100 µM AP for 2 hr at 30° and efflux of AP was estimated as in Fig. 3 at 21°.

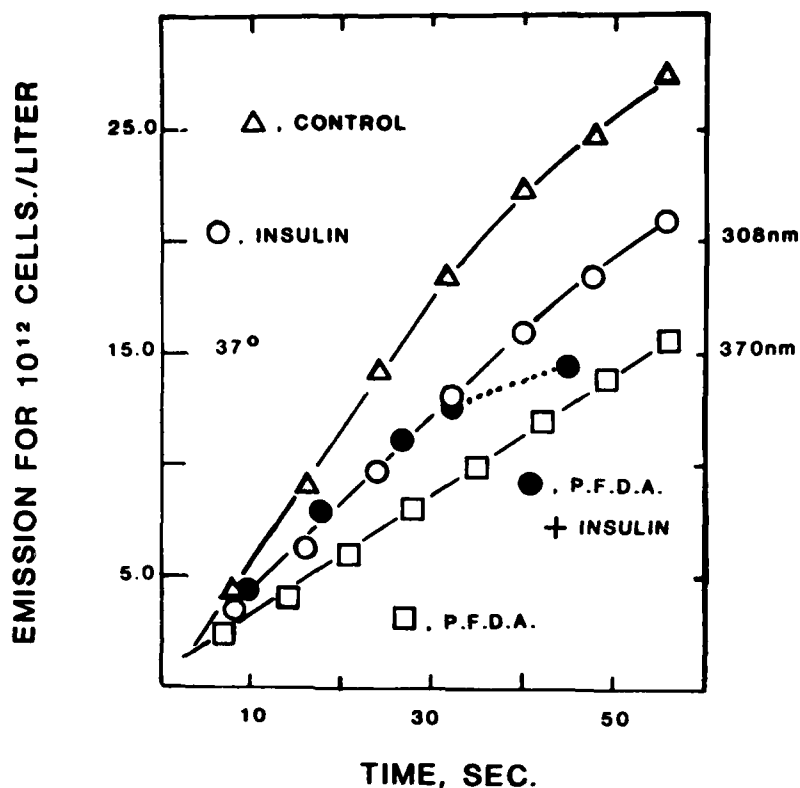


Fig. 5. Indirect Effect of a prior incubation of L5178Y Cells with 100 $\mu\text{g/ml}$ PFDA in medium for 24 hr at 37° on the Efflux of AP. Cells were incubated with PFDA (\square), 100 mU/ml bovine insulin (\circ), PFDA plus insulin (\bullet), and no additions (Δ). The cells were preloaded with 100 μM AP and the efflux rate was determined with the filtration-flow apparatus at 21° . Excitation was at 308 nm and emission was at 370 nm.

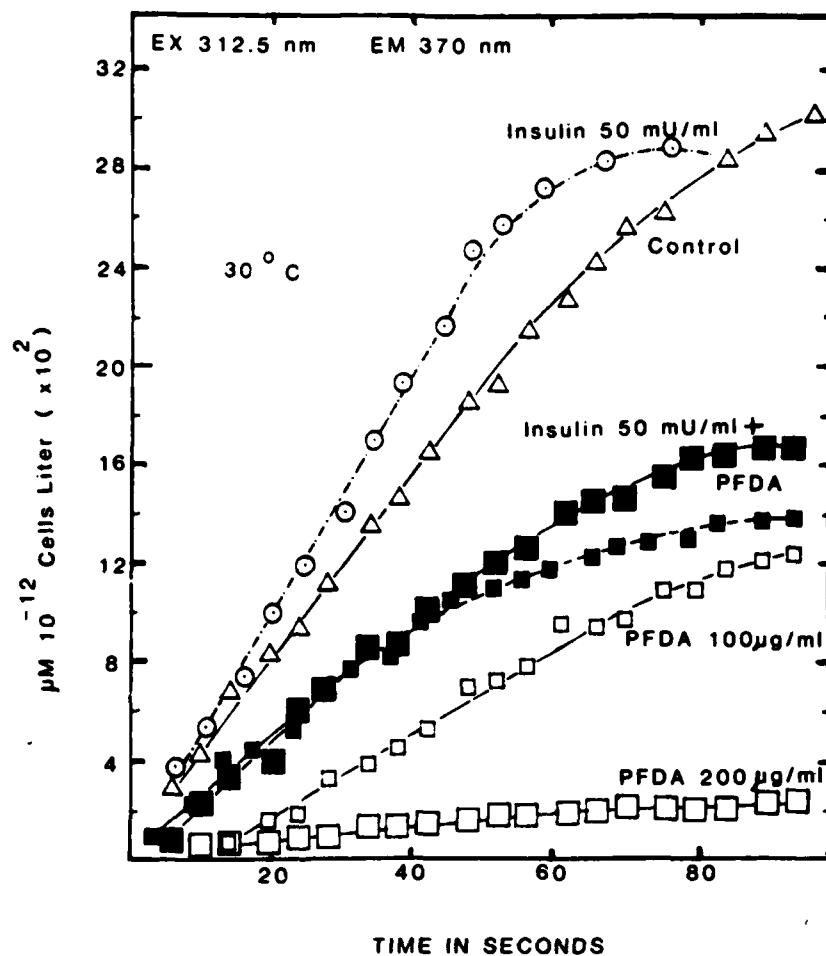


Fig. 6. Indirect Effect of a prior incubation of L5178Y Cells with PFDA in medium for 24 hr at 30°, on the Efflux of AP. Cells were incubated with 100 μg/ml PFDA (□), 200 μg/ml PFDA (□), 50 mU/ml bovine insulin (○), insulin plus 100 μg/ml PFDA (■), insulin plus 200 μg/ml PFDA (■), and no additions (Δ). The cells were preloaded with 100 μM AP and the efflux rate was determined at an excitation of 312.5 nm and an emission of 370 nm. The concentration of AP in the external buffer, for count of 10^{12} cells per liter, was plotted against time.

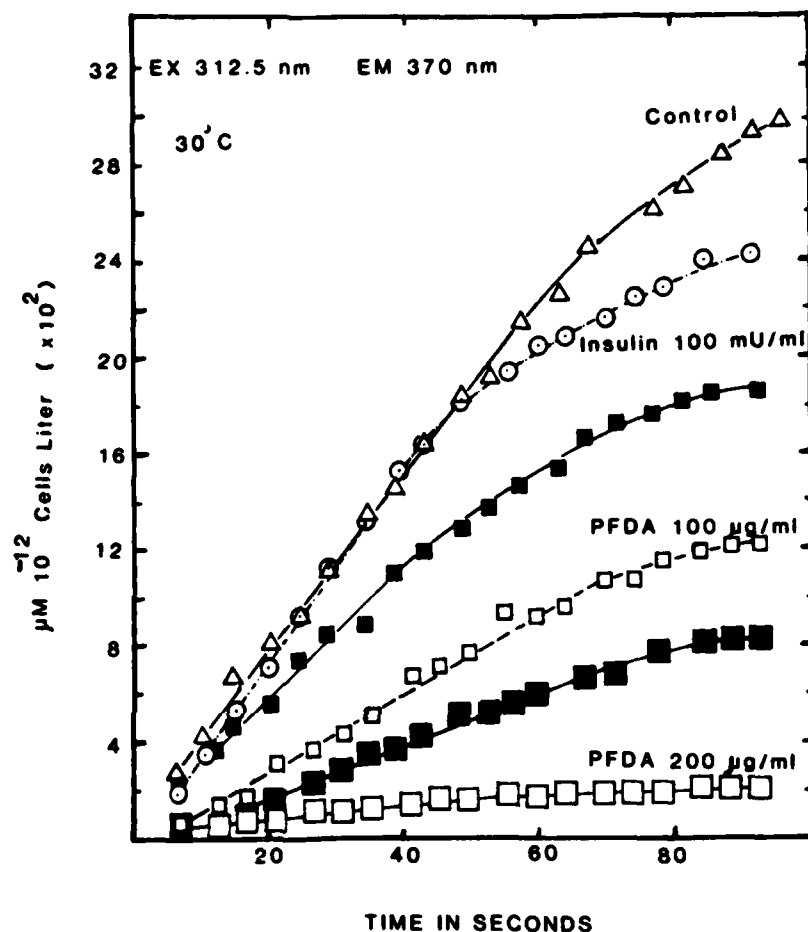


Fig. 7. Indirect Effect of a Prior Incubation of L5178Y Cells with PFDA in medium for 24 hr at 30°, on the Efflux of AP. Cells were incubated with 100 μg/ml PFDA (□), 200 μg/ml PFDA (□), 100 mU/ml bovine insulin (○), insulin plus 100 μg/ml PFDA (■), insulin plus 200 μg/ml PFDA (■), and no additions (Δ). The cells were preloaded with AP and the efflux rate was determined at an excitation of 312.5 nm and an emission of 370 nm. The concentration of AP in the external buffer, for a count of 10¹² cells per liter, was plotted against time.

HYPOTHESIS

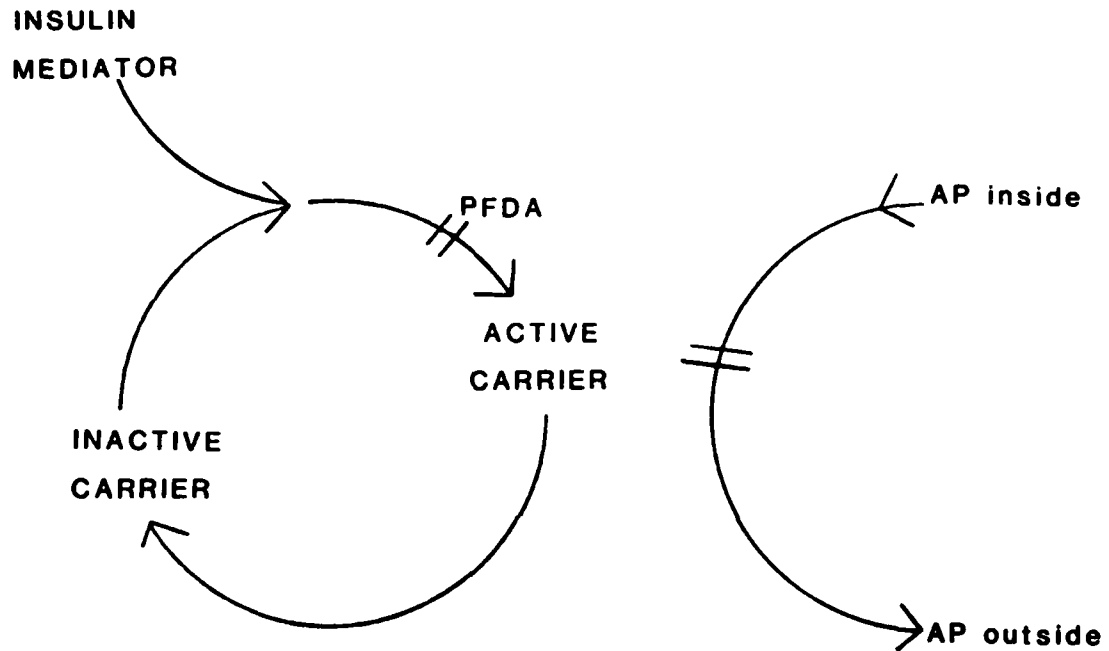


Fig. 8. A Hypothetical Scheme for the Mechanism of PFDA and Insulin Action on the Carrier for 2-Aminopurine. The carrier may exist in an active and an inactive form with turnover mediated by lipid and protein synthesis in the plasma membrane. PFDA inhibits the conversion of inactive carrier to active carrier. Insulin interacts with the insulin receptor to produce an insulin mediator. The mediator reacts with the carrier to protect the carrier from PFDA action.

EFFECTS OF PERFLUORODECANOIC ACID ON TRANSPORT IN L5178Y CELLS

Paul W. Wigler and Yatish B. Shah

University of Tennessee Memorial Research Center
Knoxville, Tennessee 37920

Treatment of L5178Y mouse lymphoma cells with perfluorodecanoic acid (PFDA) produces alterations in the structure of the plasma membrane at the cell surface (Andersen, M.E., A.M. Rogers, M.E. George, and K.C. Back, AFAMRL-TR-82-00). Would PFDA treatment modify the mechanism of flux through the cell membrane for a fluorescent substrate? Hypotheses about the kinetics of nucleoside and purine transport are based on a mechanism for substrate flux:



$$K_S = \frac{(1 + h/g) (bd + be + ce)}{a(c + d + e + ce/g)} \quad V_S = \frac{ce [C_t]}{c + d + e + ce/g} \quad (2)$$

Mechanism 1 shows the efflux of a fluorescent substrate from a pre-loaded cell into the external buffer; the rate constants for the forward reaction are a, c, e, and g and the reverse constants are h, d, and b. The symbol S_i represents the intracellular substrate and S_o represents the substrate in the external buffer. $[C_t]$ is the sum of the concentrations of the different carrier species C_i , CS_i , CS_o , and C_o . The rate equation for zero-*trans* efflux, in the form of the Michaelis-Menten equation, gives the Michaelis constant (K_S) and maximum velocity (V_S) shown in expression 2. If a, b, or h is influenced by the PFDA treatment of L5178Y cells and the other rate constants are unaffected, the K_S of the efflux reaction would change but the V_S would not.

The L5178Y cells are incubated in PFDA and McCoy's 5A medium for 24 hr at 37°; controls receive no PFDA treatment. The cells are then preloaded at 30° with 2-aminopurine (AP, a fluorescent substrate), cooled to 4°, and centrifuged at 4°. The cell pellets are resuspended and centrifuged twice in cold phosphate-buffered saline (PBS). The pellets are resuspended in PBS at 20° and aliquots are removed at 10 sec intervals. Each aliquot is added to a cold quench solution, centrifuged, and the cells are discarded. The AP content of each supernatant, containing 0.02 to 0.1 μ M AP, is estimated (at an excitation of 308 nm and an emission of 370 nm) with a Spectrofluorometer, an Hg-Xe lamp, and a 9635QA photomultiplier. A continuous flow system for efflux rates is under construction.

The chemical synthesis of another fluorescent substrate (5'-chloro, 5'-deoxyformycin; CldF) is in progress. Substitution of a chlorine atom at the 5'-position of formycin prevents nucleotide formation in L5178Y cells, and the presence of a carbon-carbon bond at the 1'-position prevents enzymic cleavage of the glycosyl bond. Hexa-methylphosphoric triamide is mixed with $SOCl_2$, formycin, and N_2 gas; the mixture is stirred for several hr at ambient temperature. Crude CldF is purified with a column of Bio-Rad Ag 50W-X8 (K^+ form, minus 400 mesh). The effect of PFDA treatment on the efflux rate of CldF from L5178Y cells will be determined (Supported by research grant AFOSR-82-0261).

(To be filled in by Division)

- A.** DIVISION OF Symposium on "Structure and Transport in Membranes"
B. TITLE OF PAPER Toxicant Effects on the Efflux Rate of Fluorescent Substrates from L5178Y Cells

Paper number as listed on program _____

Time Required for Presentation _____

☐ Poster Presentation Preferred

- C.** AUTHORS
Underline name of speaker

Paul W. Wigler
Yatish B. Shah

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Memorial Research Center
1924 Alcoa Highway
Knoxville, TN 37920
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TOXICANT EFFECTS ON THE EFFLUX RATE OF FLUORESCENT SUBSTRATES FROM L5178Y CELLS
Paul W. Wigler and Yatish B. Shah, University of Tennessee Memorial Research Center, Knoxville, Tennessee 37920

The exposure of an animal cell to a toxicant may damage the carriers in the cell membrane. L5178Y mouse lymphoma cells were incubated in perfluorodecanoic acid (PFDA) and growth medium; the controls received no PFDA treatment. The mouse cells were then preloaded with 2-aminopurine (AP), a fluorescent substrate. The cell suspension was then cooled to 4° and the preloaded cells were resuspended twice in cold phosphate-buffered saline (PBS). The cell pellet was resuspended in PBS at 25° and the cell suspension was placed over a 5.0 µm membrane filter. The effluent solution was drained through a quartz flow cell inside a fluorescence spectrophotometer. Light from an Hg-Xe lamp at 307 nm was used for excitation, and the AP emission at 370 nm was estimated at 10 sec intervals with a 9635QA photomultiplier. Preliminary results indicate that the initial rate of AP efflux from PFDA-treated L5178Y cells is less than the AP efflux rate from controls. (Supported by Research Grant 82-0261 from the Air Force Office of Scientific Research.)

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INSTITUTE OF BIO-ORGANIC CHEMISTS

August 15, 1983

Dr. Paul W. Wigler
The University of Tennessee
Department of Medical Biology
Memorial Research Center
1924 Alcoa Highway
Knoxville, Tennessee 37920

Dear Dr. Wigler:

In response to your earlier request, enclosed is a sample of 9-(1,3-dihydroxy-2-propoxymethyl)-2-aminopurine for use in your studies on the kinetics of cell membrane transport. We apologize for the delay in sending this to you, but in addition to the lag in preparing the paperwork a new synthesis had to be undertaken since our supplies of this compound had been used up.

I look forward to hearing of your results.

Sincerely yours,

Handwritten signature of John C. Martin in cursive script.

John C. Martin
Staff Researcher II

:mb

END

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